

LOW OXYGEN AFFINITY MUTANT HEMOGLOBINS

ACKNOWLEDGMENT

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FIELD OF THE INVENTION

This invention relates generally to novel mutant hemoglobins and more particularly relates to recombinant mutant hemoglobins "rHb (β N108Q)" (alternative designation "rHb (β 108Asn \rightarrow Gln)") and "rHb (β L105W)" (alternative designation "rHb (β 105Leu \rightarrow Trp)") that possess low oxygen affinity, and high cooperativity in oxygen binding. In particular, rHb (β N108Q) exhibits increased resistance to autoxidation as compared to other known low oxygen affinity mutants. This invention further relates to the preparation of mutant hemoglobins using recombinant DNA technology that are useful as substitutes for red blood cells and for hemoglobin-based therapeutics.

BACKGROUND OF THE INVENTION

The prevalence of infectious agents such as HIV and hepatitis in red blood cells of human blood products coupled with blood shortages from lack of suitable donors has led to great interest in the development of red blood cell substitutes, particularly human hemoglobin ("Hb") and its derivatives. Hemoglobin-based oxygen carriers are potential sources of blood substitutes during emergency medical situations.

See, for example, Winslow, R.M., et al. Hemoglobin-Based Red Cell Substitutes, Johns

Hopkins University Press, Baltimore (1992) (hereinafter "Winslow, et al. (1992)"), the disclosure of which is incorporated herein by reference.

Hemoglobin is the oxygen-carrying component of blood, circulated through the blood stream inside erythrocytes (red blood cells). Human normal adult hemoglobin ("Hb A") is a tetrameric protein with a molecular weight of about 64,500 containing two identical α chains having 141 amino acid residues each and two identical β chains having 146 amino acid residues each, with each also bearing prosthetic groups known as hemes. The erythrocytes help maintain hemoglobin in its reduced, functional form. The heme-iron atom is susceptible to oxidation, but may be reduced again by one of two systems within the erythrocyte, the cytochrome b₅, and glutathione reduction systems. For a review on hemoglobin, see, Dickerson, R.E., et al. Hemoglobin: Structure, Function, Evolution, and Pathology, Benjamin/Cummings, Menlo Park, CA (1983) (hereinafter "Dickerson, et al. (1983)"), the disclosure of which is incorporated herein by reference.

The oxygenation process of Hb A is cooperative, i.e., the binding of the first oxygen molecule enhances the binding of the second, third, and fourth oxygen molecules. The oxygenation process is also regulated by interactions between individual amino acid residues and various solutes, known as heterotropic allosteric effectors. These effectors include ions or molecules such as hydrogen ion, chloride, carbon dioxide, inorganic phosphate, and organic polyanions, such as 2,3-bisphosphoglycerate ("2,3-BPG") and inositol hexaphosphate ("IHP").

Hemoglobin is able to alter its oxygen affinity, thereby increasing the efficiency of oxygen transport in the body, due to its dependence on the allosteric effector 2,3-BPG. 2,3-BPG is present within erythrocytes at a concentration that allows hemoglobin to release bound oxygen to tissues. In the absence of 2,3-BPG, hemoglobin
5 binds oxygen very tightly and does not readily release its bound oxygen. The Hb A molecule alone, were it to be introduced into a subject, would not be able to properly allow oxygen to be delivered to tissues in the body due to a lack of 2,3-BPG, which lowers the oxygen affinity of Hb, in the blood plasma. See, Winslow, et al. (1992). Any Hbs designed to be functional as Hb-based oxygen carriers or hemoglobin therapeutics
10 should be able to deliver oxygen efficiently, i.e., they should load and unload cooperatively as Hb A does inside red blood cells.

The use of cell-free solutions of hemoglobin as a potential oxygen-carrying red cell substitute has been investigated for a long time. See, for example, Mulder, A.G., et al., J. Cell Comp. Physiol. 5:383 (1934), the disclosure of which is incorporated herein
15 by reference. However, the use of unmodified cell-free human hemoglobin purified from red blood cells suffers from several limitations in addition to contamination and supply limitations noted above, namely, an increase in oxygen affinity due to loss of allosteric effectors, such as 2,3-BPG, and dissociation of Hb tetramers into $\alpha\beta$ dimers which are cleared by renal filtration and which can cause long-term kidney damage.
20 See, for example, Bunn, H.F., et al. J. Exp. Med. 129:909 (1969), the disclosure of which is incorporated herein by reference.

Human globins and hemoglobins have been expressed in the following:
transgenic mice, see, for example, Chada, K., et al., Nature (London) 314:377 (1985) and
Townes, T. M., et al. EMBO J. 4:1715 (1985), transgenic swine as described by Swanson,
M.E., et al. Bio/Technology 10:557 (1992), insect cell cultures as reported by Groebe,
5 D.R., et al., Protein Expression and Purification 3:134 (1992), yeast as described by
Wagenbach, M., et al. Bio/Technology 9:57 (1991) and DeLiano, J.J., et al. Proc. Natl.
Acad. Sci. USA 90:918 (1993), and Escherichia coli ("E. coli") as described by Hoffman,
S.J., et al. Proc. Natl. Acad. Sci. USA 87:8521 (1990), Hernan, R.A., et al. Biochemistry
31:8619 (1992), and Shen, T.-J., et al. Proc. Natl. Acad. Sci. USA 90:8108 (1993)
10 (hereinafter "Shen, et al. (1993)"), all the disclosures of which are incorporated herein by
reference. In many respects, the E. coli system is the best choice for such purposes
because of its high expression efficiency and the ease of performing site-directed
mutagenesis.

The natural N-terminal valine residues of Hb A are known to play
15 important roles in regulating oxygen affinity, the Bohr effect, and interactions with
allosteric effectors and anions as reported by Bunn, H.F., et al. eds. Hemoglobin:
Molecular, Genetic and Clinical Aspects (W.B. Saunders, Co., Philadelphia, PA) pp. 37-
60 (1986) (hereinafter "Bunn, et al. (1986)"), the disclosure of which is incorporated
herein by reference. The extra methionine can alter the N-terminal conformation of the
20 Hb molecule as reported by Kavanaugh, J.S., et al. Biochemistry 31:8640 (1992), the
disclosure of which is incorporated herein by reference. Hence, the oxygenation
properties of Hb depend on the integrity of the N-terminal residue thereby mandating

the removal of the extra methionine residues from the N-termini of both the α - and β -globins of the expressed Hb before the E. coli system can be used effectively for the production of desired unmodified and mutant Hbs.

The cooperative oxygenation of Hb, as measured by the Hill coefficient ("n_{max}") is a convenient measure of its oxygenation properties. See, Dickerson, et al. (1983). Hb A has an n_{max} value of approximately 3 in its binding with O₂ under usual experimental conditions. Human abnormal Hbs with amino acid substitutions in the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) subunit interface generally result in high oxygen affinity and reduced cooperativity in O₂ binding compared to Hb A. See, for example, Dickerson, et al. (1983); Bunn, et al (1986) and Perutz, M.F., et al. Mechanisms of Cooperativity and Allosteric Regulation in Proteins Cambridge University Press (1990), the disclosure of which is incorporated herein by reference.

Hb A in its oxy form (Hb A with oxygen molecules) has a characteristic hydrogen bond between $\alpha 94\text{Asp}$ and $\beta 102\text{Asn}$ in the $\alpha_1\beta_2$ subunit interface as reported by Shaanan, B., et al. J. Mol. Biol. 171:31 (1983), the disclosure of which is incorporated herein by reference (hereinafter "Shaanan, et al. (1983)"). Human Hbs with an amino acid substitution at either the $\alpha 94\text{Asp}$ position such as Hb Titusville ($\alpha 94\text{Asp} \rightarrow \text{Asn}$) (Schneider, R. G., et al. Biochim. Biophys. Acta. 400:365 (1975), the disclosure of which is incorporated herein by reference) or the $\beta 102\text{Asn}$ position such as Hb Kansas ($\beta 102\text{Asn} \rightarrow \text{Thr}$) (Bonaventura, J., et al. J. Biol. Chem. 243:980 (1968), the disclosure of which is incorporated herein by reference), as well as others with mutations in the $\alpha_1\beta_2$ subunit interface, exhibit very low oxygen affinity. However, all these Hb mutants

which directly disrupt the hydrogen bond between $\alpha 94$ Asp and $\beta 102$ Asn in the oxy form of Hb show greatly reduced cooperativity in the binding of oxygen and additionally dissociate easily into dimers when in the ligated state.

It has also been shown that during the transition from the deoxy-to the oxy-state, the $\alpha_1\beta_2$ subunit of Hb A undergoes a sliding movement, while the $\alpha_1\beta_1$ subunit interface remains nearly unchanged (See, Perutz, M.F. Nature 228: 726 (1970) ("Perutz (1970)"); Baldwin, J.M., et al. J. Mol. Biol. 129: 175 (1979); Baldwin, J.M., J. Mol. Biol. 136: 103 (1980); Shaanan, et al. (1983); and Fermi, G., et al. J. Mol. Biol. 175: 159 (1984), ("Fermi, et al., (1984)"), the disclosures of which are incorporated herein by reference. There are specific hydrogen bonds, salt bridges, and non-covalent interactions that characterize both subunit interfaces. The Hb molecule also has a lower oxygen affinity in the deoxy quaternary structure (T-structure) than in the oxy quaternary structure (R-structure) See, Dickerson, et al. (1983).

Low oxygen affinity human mutant Hbs which do not involve either $\alpha 94$ Asp or $\beta 102$ Asn also exist. For example, Hb Presbyterian ($\beta 108$ Asn \rightarrow Lys) (Moo-Penn, W.F., et al. FEBS Lett. 92:53 (1978) and O'Donnell, J.K., et al. J. Biol. Chem. 269:27692 (1994) (hereinafter "O'Donnell, et al. (1994)"); Hb Yoshizuka ($\beta 108$ Asn \rightarrow Asp), O'Donnell, et al. (1994) and recombinant Hb Mequon ($\beta 41$ Phe \rightarrow Tyr) (Baudin, V., et al. Biochim. Biophys. Acta. 1159:223 (1992), the disclosures of which are incorporated herein by reference, all exhibit low oxygen affinity compared to Hb A, but they all exhibit a variable amount of cooperativity as measured by the Hill coefficient, with n varying from 1.8 to 2.9. Tsai, C.-H., et al. Biochemistry 38:8751 (1999) (hereinafter, "Tsai,

et al. (1999)") report Hb ($\alpha 96\text{Val}\rightarrow\text{Trp}$, $\beta 108\text{Asn}\rightarrow\text{Lys}$) which has low oxygen affinity and a greater tendency to switch to the T quaternary structure. Jeong, S.T., et al., Biochemistry 38:13433 (1999) (hereinafter, "Jeong, et al. (1999)") report that Hb ($\alpha 29\text{Leu}\rightarrow\text{Phe}$, $\alpha 96\text{Val}\rightarrow\text{Trp}$, $\beta 108\text{Asn}\rightarrow\text{Lys}$) exhibits low oxygen affinity and high cooperativity combined with resistance to autoxidation.

Shen, et al. (1993) and U.S. Patent No. 5,753,465, the disclosures of which are incorporated herein by reference, describe an E. coli expression plasmid (pHE2) in which synthetic human α - and β -globin genes are coexpressed with the E. coli methionine aminopeptidase gene under the control of separate tac promoters. E. coli cells transformed with this plasmid express recombinant Hb A (hereinafter "rHb A") from which the N-terminal methionines have been effectively cleaved by the coexpressed methionine aminopeptidase. The resulting rHb A which lacks an N-terminal methionine is identical to the native Hb A in a number of structural and functional properties.

Kim, H.-W., et al. Proc. Natl. Acad. Sci. USA 91:11547 (1994) (hereinafter "Kim, et al. (1994)"), and U.S. Patent No. 5,843,888, the disclosures of which are incorporated herein by reference, describe a non-naturally occurring mutant hemoglobin (rHb ($\alpha 96\text{Val}\rightarrow\text{Trp}$) (alternative designation "rHb (αV96W)") that has a lower oxygen affinity than that of native hemoglobin, but high cooperativity in oxygen binding.

There remains a need, however, for additional mutant hemoglobin species that can be used as a component of a hemoglobin-based blood substitute or therapeutic

agent. Of particular interest is a mutant hemoglobin that possesses low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation.

There is a further need for such a hemoglobin produced by recombinant methods and an efficient expression system for producing such a mutant hemoglobin in high yield,

5 especially for use in a blood substitute product or hemoglobin therapeutics.

SUMMARY OF THE INVENTION

Accordingly, it is a primary object of the present invention to provide mutant human hemoglobins with low oxygen affinity and high cooperativity in oxygen binding.

10 Another object of the present invention is to provide mutant hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation.

Another object of the present invention is to provide non-naturally occurring mutant human hemoglobins with low oxygen affinity and high cooperativity
15 in oxygen binding.

Another object of the present invention is to provide non-naturally occurring mutant human hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and increased stability against autoxidation.

Another object of the present invention is to provide non-naturally
20 occurring mutant human hemoglobins with low oxygen affinity, high cooperativity in oxygen binding, and preferably with stability against autoxidation that are produced

artificially, preferably by recombinant means, and that have the correct heme conformation.

Another object of the present invention is to provide mutant hemoglobins that in a cell-free environment have similar oxygen binding properties as those of human normal adult hemoglobin in red blood cells.

Yet another object of the present invention is to provide mutant hemoglobins with low oxygen affinity and high cooperativity in oxygen binding in which the T-structure is stabilized while the R-structure is undisturbed.

Still another object of the present invention is to provide artificial hemoglobins for use as a hemoglobin-based oxygen carrier/red blood substitute or therapeutic agent.

These and other objects of the present invention are achieved by one or more of the following embodiments.

In one aspect, the invention features a non-naturally occurring mutant human hemoglobin wherein the asparagine residue at position 108 of the β -chains is replaced by a glutamine residue.

In a preferred embodiment, the hemoglobin possesses low oxygen affinity as compared to human normal adult hemoglobin, high cooperativity in oxygen binding, increased stability against autoxidation, and is produced recombinantly.

In another aspect, the invention features an artificial mutant hemoglobin which in a cell-free environment has oxygen binding properties comparable to those of

human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the asparagine residue at position 108 of the β -chains is glutamine.

A non-naturally occurring low oxygen affinity mutant hemoglobin with increased stability against autoxidation that has oxygen binding properties comparable
5 to those of human normal adult hemoglobin in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the asparagine residue at position 108 of each of the β -chains is replaced by a glutamine residue.

In yet another aspect, the invention features a non-naturally occurring mutant human hemoglobin wherein the leucine residue at position 105 of the β -chains
10 is replaced by a tryptophan residue.

In a preferred embodiment, the hemoglobin possesses low oxygen affinity as compared to human normal adult hemoglobin, high cooperativity in oxygen binding, and is produced recombinantly.

In another aspect, the invention features an artificial mutant hemoglobin
15 which in a cell-free environment has oxygen binding properties comparable to those of human normal adult hemoglobin in red blood cells wherein said hemoglobin contains a mutation such that the leucine residue at position 105 of the β -chains is tryptophan.

A non-naturally occurring low oxygen affinity mutant hemoglobin that has oxygen binding properties comparable to those of human normal adult hemoglobin
20 in the presence of the allosteric effector 2,3-bisphosphoglycerate, wherein the leucine residue at position 105 of each of the β -chains is replaced by a tryptophan residue.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1A is a cDNA sequence (SEQ ID NO: 5) for the alpha- and beta-globin genes for rHb (β N108Q) derived from plasmid pHE7009.

Figure 1B is a cDNA sequence (SEQ ID NO: 7) for alpha- and beta-globin genes for rHb (β L105W) derived from plasmid pHE7004.

Figures 2A and 2B show the FPLC profiles of rHb (β N108Q) (peak b) (Figure 2A) and rHb (β L105W) (peak b) (Figure 2B).

Figures 3A and 3B show the pH dependence of the oxygen affinity (P_{50}) and the Hill coefficient (n_{max}), respectively, of rHb (α L29F) (\square); rHb (β N108Q) (Δ); rHb (α L29F, β N108Q) (\times); rHb (β L105W) (\blacktriangle); and Hb A (O) in 0.1 M sodium phosphate buffer at 29°C. Oxygen dissociation data were obtained with 0.1 mM Hb.

Figure 4 shows oxygen-binding curves of rHb (β N08Q); rHb (α L29F, β N108Q); rHb (β L105W); and Hb A with and without the presence of allosteric effector, 5 mM 2, 3- BPG, in 0.1 M phosphate buffer at pH 7.4 and 29°C. Protein concentration was 0.1 mM heme.

Figure 5 shows the autoxidation of Hb A (O); rHb (β N108Q) (\blacktriangle); rHb (β L105W) (Δ); rHb (α V96W) (∇); rHb (α V96W, β N108K) (\blacktriangledown); rHb (α L29F, β N108Q) (\diamond); and rHb (α L29F, α V96W, β N108K) (\square) in PlasmaLyte buffer in the presence of 5 mM EDTA and 5% D₂O at pH 7.4 and 37°C. The autoxidation process was measured by

monitoring the rate of disappearance of the oxy-marker at -2.34 ppm upfield from DSS by 300-MHz ^1H -NMR.

Figures 6A and 6B are 500-MHz ^1H -NMR spectra showing exchangeable proton resonances (Figure 6A) and ring-current shifted proton resonances (Figure 6B), respectively, of Hb A; rHb (βN108Q), and rHb (αL29W , βL108Q), all in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 and 29°C.

Figures 7A and 7B are 300-MHz ^1H -NMR spectra showing ferrous hyperfine-shifted N_δH resonances of proximal histidines and hyperfine-shifted and exchangeable proton resonances, respectively, of rHb A; rHb (αL29F); rHb (βN108Q); and rHb (αL29W , βN108Q), all in the CO form, in 0.1 M sodium phosphate buffer at pH 7.0 and 29°C.

Figures 8A and 8B are 500-MHz spectra showing the exchangeable proton resonances of rHb (βN108Q) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 at 500 MHz at various temperatures (7°C, 11°C, 17°C, 23°C, 29°C), without an allosteric effector (Figure 8A) and with 4 mM inositol hexaphosphate ("IHP") (Figure 8B).

Figures 9A and 9B are 500-MHz spectra showing the exchangeable proton resonances of rHb (αL29F , βN108Q) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 at various temperatures (7°C, 11°C, 17°C, 23°C, 29°C), without (Figure 9A) and with (Figure 9B) 4 mM IHP.

Figures 10A and 10B are 600-MHz ^1H -NMR spectra showing exchangeable proton resonances (Figure 10A) and ring-current shifted proton

resonances (Figure 10B) of 3-6% solutions of Hb A; rHb (β L105W); rHb (α D94A, β L105W); and rHb (α D94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 and 29°C.

Figures 11A and 11B are 600-MHz 2D heteronuclear multiple-quantum coherence ("HMQC") spectra of 5-8% solutions of ^{15}N -labeled rHb (β L105W) (Figure 11A) and Hb A (Figure 11B) in the CO form in 90% H_2O /10% D_2O in 0.1 M sodium phosphate at pH 7.0 and 29°C.

Figures 12A-12D are 600-MHz 2D NOESY-HMQC ("NOESY"-nuclear Overhauser enhancement spectroscopy) spectra of a 5% solution of ^{15}N -labeled rHb (β L105W) in the CO form in 90% H_2O /10% D_2O in 0.1 M sodium phosphate at pH 7.0 and 29°C recorded at various mixing times: 15 ms (Figure 12A); 30 ms (Figure 12B); 60 ms (Figure 12C); and 100 ms (Figure 12D).

Figures 13A-13C are 300-MHz ^1H -NMR spectra of 3-6% solutions of Hb A; rHb (β L105W); rHb (α D94A, β L105W); and rHb (α D94A) in the deoxy form in 0.1 M sodium phosphate at pH 7.0 and 29°C. Figure 13A shows hyperfine-shifted N_δH resonances of proximal histidines acquired at 300-MHz; Figure 13B shows hyperfine shifted and exchangeable proton resonances acquired at 300-MHz; and Figure 13C shows exchangeable proton resonances acquired at 300-MHz. Since rHb (α D94A, β L105W) and rHb (α D94A) easily form met-Hb during the oxygenization process, a small amount of sodium dithionite was added to these NMR samples to diminish the formation of met-Hb.

Figure 14 is a 600-MHz 2D HMQC spectrum of 5-8% solutions of ^{15}N -labeled rHb (βL105W) in the deoxy form in 90% H_2O /10% D_2O in 0.1 M sodium phosphate at pH 7.0 and 29°C.

Figures 15A-15D are 600-MHz 2D NOESY-HMQC spectra of 5% solution of ^{15}N -labeled rHb (βL105W) in the deoxy form in 90% H_2O /10% D_2O in 0.1 M sodium phosphate at pH 7.0 and 29°C recorded at various mixing times: 15 ms (Figure 15A); 30 ms (Figure 15B); 60 ms (Figure 15C); and 100 ms (Figure 15D). The solid line between two cross peaks indicates the inter-residue NOE effect between the $^1\text{H}_{\epsilon 1}$ of one residue and the $^1\text{H}_{\delta 1}$ and $^1\text{H}_{\zeta 2}$ of the other residue for $\beta 37\text{Trp}$ and $\beta 105\text{Trp}$.

Figures 16A-16B show exchangeable proton resonances in 600-MHz ^1H -NMR spectra of 3-6% solutions of Hb A; rHb (βL105W); rHb (αD94A , βL105W); and rHb (αD94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 and at 11°, 20°, and 29°C in the absence (Figure 16A) and presence (Figure 16B) of 2 mM IHP.

Figure 17A-17D are 600-MHz 2D heteronuclear single-quantum coherence ("HSQC") spectra of 5-8% solutions of ^{15}N -labeled rHb (βL105W) in the CO form in 90% H_2O /10% D_2O in 0.1 M sodium phosphate at pH 7.0 and various temperatures in the absence and presence of 2 mM IHP. Figure 17A - 29°C in the absence of IHP; Figure 17B - at 29°C in the presence of 2 mM IHP; Figure 17C - at 20°C in the presence of 2 mM IHP; and Figure 17D - 11°C in the presence of 2 mM IHP.

Figures 18A-18B show ring-current shifted proton resonances in 600-MHz ^1H -NMR spectra of 3-6% solutions of Hb A; rHb (βL105W); rHb (αD94A , βL105W); and

rHb (α D94A) in the CO form in 0.1 M sodium phosphate at pH 7.0 and various temperatures in the absence (Figure 18A) and presence (Figure 18B) of 2 mM IHP.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

As used herein, "Hb A" or "native Hb A" means human normal adult hemoglobin as obtained from human subjects.

"Recombinant human normal adult hemoglobin," "rHb A," and "unmodified rHb A" mean human normal adult hemoglobin produced through recombinant DNA technology and having essentially the same structure and function as native Hb A as described by Shen, et al. (1993), and U.S. Patent No. 5,753,465.

"rHb (β L105W)" refers to a recombinant mutant human hemoglobin in which the leucine residue at position 105 of each of the β -chains of the Hb molecule has been replaced by a tryptophan residue. This hemoglobin possesses low oxygen affinity and high cooperativity in oxygen binding compared to Hb A. rHb (β L105W) is designed to form a hydrogen bond between α 94Asp and β 105Trp in the $\alpha_1\beta_2$ subunit interface in order to lower the oxygen affinity by stabilizing its deoxy quaternary structure.

"rHb (β N108Q)" refers to a mutant human hemoglobin produced through recombinant DNA technology in which the asparagine residue at position 108 of the β -chains located in the $\alpha_1\beta_1$ interface and in the central cavity of the Hb molecule, have been replaced by glutamine residues. This hemoglobin possesses low oxygen affinity, and high cooperativity in oxygen binding, and also increased resistance to autoxidation

as compared to other known recombinant low oxygen affinity mutant hemoglobins, such as, rHb (α V96W) and rHb (α V96W, β N108K).

"Autoxidation" refers to the turning or conversion of oxyhemoglobin ("HbO₂" or "oxy-Hb") into methemoglobin ("met-Hb"). In HbO₂ the heme-iron atoms are in the reduced ferrous (Fe²⁺) state, however, in met-Hb, the heme-iron atoms are in the oxidized ferric (Fe³⁺) state.

"Deoxy" and "oxy" refer to the oxygenation state of the heme-iron atom in Hb A and rHbs. Oxyhemoglobin ("oxy-Hb" or "HbO₂") has four oxygen molecules bound to the heme groups; deoxyhemoglobin ("deoxy-Hb") contains no oxygen molecules. In normal arterial blood, normal adult hemoglobin A ("Hb A") is in the oxy form ("Hb O₂ A" or "oxy-Hb A"). In venous blood, a portion of Hb A is in the deoxy form ("deoxy-Hb A").

"Carbonmonoxy-Hb," "HbCO A," "rHbCO," and "CO form" all refer to hemoglobin bound to carbon monoxide molecules rather than oxygen molecules.

"Ferri-hemoglobin," "ferri-Hb," "ferric form," "methemoglobin," "met-Hb", and "Fe⁺³ state" all refer to hemoglobin with their respective heme-iron atoms oxidized to the ferric (Fe³⁺) state. Ferri-Hb does not bind oxygen.

"Methionine aminopeptidase" refers to the enzyme methionine aminopeptidase which specifically cleaves the amino-(N) terminal methionine residue from a peptide sequence.

"Oxygen affinity" means the strength of binding of oxygen to a hemoglobin molecule. High oxygen affinity means hemoglobin does not readily release its bound oxygen molecules. The P_{50} is a measure of oxygen affinity.

"Cooperativity" refers to the binding of oxygen by the four subunits of the hemoglobin molecule and is measured by the Hill coefficient (n_{\max}). For Hb A in 0.1 M sodium phosphate at pH 7.4 and 29°C, n_{\max} is about 3.2.

The two classical quaternary structures are the T (tense) quaternary D structure for the low affinity deoxy-Hb and the R (relax) quaternary structure for the high affinity oxy-Hb. "R-type" or "R-like," and similar terms refer to those hemoglobins which exhibit characteristic quaternary structural markers, such as the proton resonance at 10.7 ppm from DSS on ^1H -NMR spectra. "T-type" or "T-like" and similar terms refer to those hemoglobins which exhibit characteristic T quaternary structures, such as the proton resonance at ~14.0 ppm from DSS on ^1H -NMR spectra.

II. METHODS AND RESULTS

Using the Escherichia coli expression system described by Shen, et al. (1993); U.S. Patent No. 5,753,465; and Kim, et al. (1995); U.S. Patent No. 5,843,888, new non-naturally occurring artificial recombinant hemoglobins ("rHbs") have been constructed, having low oxygen affinity while maintaining high cooperativity in oxygen binding. One of the rHbs, rHb (βN108Q) also exhibits increased resistance to autoxidation as compared to certain other known low oxygen affinity mutants. More particularly, the present invention is directed to: a recombinantly produced mutant of Hb A, denoted herein as rHb (βN108Q), in which the asparagine residues at position

108 of each of the β -chains, located in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the Hb molecule, have been replaced by glutamine residue; and a recombinantly produced mutant of Hb A, denoted herein as rHb (β L105W) in which the leucine residues at position 105 of each of the β chains have been replaced by tryptophan and in this molecule a new hydrogen bond is formed from β 105Trp to β 94Asp in the $\alpha_1\beta_2$ subunit interface in order to lower the oxygen binding affinity by stabilizing its deoxy quaternary structure.

These new artificial hemoglobins, i.e., derived entirely from sources other than blood, possess a low oxygen affinity and high cooperativity in oxygen binding.

Additionally, rHb (β N108Q) exhibits increased resistance to autoxidation as compared to other known low oxygen affinity mutants, such as rHb (α V96W) and rHb (α V96W, β N108K). Further, these new artificial hemoglobins exhibit no unused subunit disassociation when ligated. In a cell-free environment the rHbs of the present invention have similar or lower oxygen binding properties to those of Hb A in red blood cells. Such rHbs therefore are of value as hemoglobin-based oxygen carriers, i.e., potential blood substitutes, or hemoglobin therapeutics.

It is also within the scope of the present invention to prepare and use other low oxygen affinity hemoglobins with other appropriate mutations. In particular, the methods of the present invention may be used to produce other mutant hemoglobins with additional advantageous properties. Methods for evaluating the suitability of other useful mutants possessing the properties of such low oxygen affinity, high cooperativity, and increased resistance to autoxidation for use in a blood

substitute or therapy are described herein below. The preferred materials and methods for obtaining rHb (β L105W) and rHb (β N108Q) are given in the following reference examples. While the rHbs of the present invention are preferably produced recombinantly, it is understood that non-recombinant methods may also be used.

5 The preferred mutant rHbs of the present invention, rHb (β L105W) and rHb (β N108Q), can switch from the R quaternary structure to the T quaternary structure in their ligated state upon the addition of an allosteric effector, IHP, and/or by lowering the temperature. The recombinant hemoglobins of the present invention can therefore be used to gain new insight regarding the nature of subunit interactions in the $\alpha_1\beta_2$ and $\alpha_1\beta_1$ interfaces and the molecular basis for the allosteric mechanism of hemoglobin.

10 As will be shown below, rHb (β N108Q) of the present invention shows a low oxygen affinity, an enhanced Bohr effect, but a similar cooperativity as that of Hb A, and also exhibits slower autoxidation to methemoglobin ("met-Hb") as compared to other known low oxygen affinity recombinant hemoglobins such as, for example, rHb (α 96Val \rightarrow Trp) and rHb (α 96Val \rightarrow Trp, β 108Asn \rightarrow Lys) (Kim, H.-W., et al. Biochemistry 35:6620-6627 (1996) (hereinafter "Kim, et al. (1996)"); Ho, C., et al. Blood Substitutes: Present and Future Perspectives of Blood Substitutes (Tsuchida, E., Ed.), Elsevier Science SA, Lausanne, Switzerland, pp. 281-296 (1998) (hereinafter "Ho, et al. (1998)"); Jeong, et al. (1999); and Tsai, et al. (1999), the disclosures of which are incorporated
15 herein by reference), oxidize much faster. Therefore, rHb (β N108Q) can be useful for hemoglobin-based oxygen carriers and hemoglobin therapeutics.

Proton nuclear magnetic resonance ($^1\text{H-NMR}$) studies show that rHb (βN108Q) has similar tertiary structure around the heme pockets and quaternary structure in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces as compared to those of Hb A. $^1\text{H-NMR}$ studies also demonstrate that rHb (βN108Q) can switch from the R quaternary structure to the T quaternary structure without changing its ligation state upon the addition of an allosteric effector, IHP, and/or by lowering the temperature. This suggests that the T quaternary structure of rHb (βN108Q) is more stable than that of Hb A. This is consistent with the molecular mechanism of low-oxygen affinity found in rHb (αV96W) (Kim, H.-W., et al., J. Mol. Biol. **248**:867 (1995) (hereinafter "Kin, et al. (1995)"); U.S. Patent No. 5,843,888) and rHb (αV96W , βN108Q) (Ho, et al. (1998); Tsai, et al. (1999)).

It has been reported by Carver, T.E., et al. J. Biol. Chem. **267**: 14443 (1992); Brantley, R.E. Jr., et al. J. Biol. Chem. **268**: 6995 (1993) (hereinafter "Brantley, et al. (1993)"; and Eich, R.F., et al. Biochemistry **35**: 6976 (1996), the disclosures of which are incorporated herein by reference, that substitution of the Leu residue for phenylalanine at the B10 position can inhibit autoxidation in myoglobin and that at the B10 position of the α -chain can lower NO reaction with deoxy- and oxy-Hb A. Reduction of the NO reaction with oxy-Hb A by appropriate mutations, i.e., αL29F , in the distal heme pocket has been associated with reduction of the hypertensive effect recorded in vivo (Doherty, D.H., et al. Nature Biotech. **16**: 672 (1998), the disclosure of which is incorporated herein by reference). Hence, as detailed below, such mutation was further introduced into βN108Q to produce a double mutant, rHb (αL29F , βN108Q). It was found that this

double mutant is more stable against autoxidation as compared to rHb (β N108Q), but exhibits comparable oxygen binding properties to those of Hb A in the presence of allosteric effector, 2 mM 2,3-BPG.

Mutant rHb (β L105W) was designed to form a new hydrogen bond from β 105Trp to α 94Asp in the $\alpha_1\beta_2$ subunit interface in order to lower the oxygen binding affinity by stabilizing its deoxy quaternary structure. It was found that rHb (β L105W) possesses a very low oxygen affinity and maintains high cooperativity ($P_{50} = 28.2$ mm Hg, $n_{\max} = 2.6$ in 0.1 M sodium phosphate at pH 7.4 and 29°C) as compared to Hb A ($P_{50} = 9.9$ mm Hg, $n_{\max} = 3.2$ in 0.1 M sodium phosphate at pH 7.4 and 29°C). Mutant rHb (α D94A, β L105W) and rHb (α D94A) were designed to provide evidence that rHb (β L105W) forms a new hydrogen bond from β 105Trp to α 94Asp in the $\alpha_1\beta_2$ subunit interface of the deoxy quaternary structure. The multinuclear, multidimensional nuclear magnetic resonance ("NMR") studies performed in accordance with the present invention in ^{15}N -labeled rHb (β L105W) have identified the resonance of the indole nitrogen-attached proton of β 105Trp for rHb (β L105W). ^1H -NMR studies on Hb A and mutant rHbs were used to investigate the structural basis for the low oxygen affinity of rHb (β L105W). NMR results show that rHb (β L105W) forms a new hydrogen bond from β 105Trp to α 94Asp in the $\alpha_1\beta_2$ subunit of the deoxy quaternary structure. It is believed that the low oxygen affinity of rHb (β L105W) is due to the formation of a new hydrogen bond between β 105Trp and α 94Asp in the deoxy quaternary structure.

Proton nuclear magnetic resonance ("NMR") spectroscopy was used to study the tertiary and quaternary structures of Hbs in solution (Ho, et al. (1992)). Several exchangeable proton resonances at ~ 15 to ~ 9 ppm from the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate ("DSS") have been characterized as intersubunit H-bonds in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in both oxy and deoxy states of Hb A. These H-bonded protons observed by NMR can be used as structural markers in functional studies. In particular, the resonance at ~ 14 ppm from DSS has been identified as the inter-subunit H-bond between $\alpha 42\text{Tyr}$ and $\beta 99\text{Asp}$ in the $\alpha_1\beta_2$ interface of deoxy-Hb A, a characteristic feature of the T-structure of Hb A (Fung, L.W.-M., et al. Biochemistry 14:2526 (1975) (hereinafter "Fung, et al. (1975)"), 1975; Russu, I.M., et al. Biochem. Biophys. Acta 914:40 (1987) (hereinafter "Russu, et al. (1987)"). By observing this T-structure marker in both the deoxy and the CO forms of Hbs under various conditions, the stability of the T-conformation can be assessed and the transition from the T- to the R-structure can be monitored.

In the present invention, the strategy for designing rHbs with low oxygen affinity and high cooperativity was to stabilize the T-structure while not perturbing the R-structure. (See, Ho, et al. (1998); Tsai, et al. (1999)). This strategy has been demonstrated in the design of rHb (αV96W), which has low oxygen affinity and normal cooperativity (Kim et al. (1995); U.S. Patent No. 5,843,888). This designed mutation is located at the $\alpha_1\beta_2$ subunit interface and in the central cavity of the Hb molecule.

According to ^1H -NMR studies, rHbCO (αV96W) can switch from the R-structure to the T-structure without changing its ligation state when the temperature is lowered and/or

when IHP, an allosteric effector, is added. The crystal structure of rHb (α V96W) in its T-state has shown a novel water-mediated H-bond between α 96Trp N ϵ ₁ and β 101Glu O ϵ ₂ in the α ₁ β ₂ subunit interface (Puius, T.A., et al. Biochemistry 37: 9258 (1998) (hereinafter "Puius", et al. (1998)"). Both ¹H-NMR studies and the crystal structure
5 indicate that the T-structure of this rHb is stabilized. In the present invention, the NMR studies have also shown that rHbCO (β N108Q) and rHbCO (β N105W) can switch to the T quaternary structure even when they are still ligated. These results suggest that the T structure of these two rHbs are more stable than that of Hb A.

As stated above, the methods of the present invention may also be used to
10 produce other mutant artificial hemoglobins with different properties as well as hemoglobins with mutations that compensate for mutants that are naturally occurring. The preferred materials and methods for obtaining rHb (β N108Q) and rHb (β L105W) are given in the following reference example. Non-recombinant methods may also be used.

REFERENCE EXAMPLE

Construction of Expression Plasmids for rHb (β N108Q) and rHb (β L105W)

The E. coli Hb A expression plasmids pHE2 and pHE7, which respectively contain human α - and β - globin genes and cDNAs, were used as the starting plasmids for expressing the mutant hemoglobins of the present invention. The construction of
20 plasmids pHE2 and pHE7 and properties of the rHb A produced thereby are fully described in Shen, et al. (1993), U.S. Patent No. 5,753,465, and Shen, T.-J., et al. Protein

Eng. 10: 1085 (1997) (hereinafter "Shen, et al. (1997)"), Kim, et al. (1994), and U.S. Patent No. 5,843,888), the disclosure of which is incorporated herein by reference.

The construction of plasmid pHE2009 for expression of mutant r Hb (β N108Q) using synthetic globin genes was carried out as follows. The plasmid pHE2 was used as the starting plasmid and an oligonucleotide of sequence 5'-CGTCTGCTGGGTCAG GTACTAGTTTGCG-3' (SEQ ID NO: 1) (mutated codon is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon) and used as a primer to introduce the mutation β N108Q into pHE2. Techniques for oligonucleotide synthesis are well known and this invention is not limited to any particular technique. The site-directed mutagenesis procedure followed the protocol of an "Altered Sites II In-Vitro Mutagenesis System" kit (Promega Corporation, Madison, WI) and the resultant plasmid pHE2009 contained the expected mutation β N108Q.

The construction of plasmids pHE2020 (mutant rHb (α D94A) and pHE2004 (mutant rHb β L105W)) using synthetic globin genes was similar to that of pHE2009, except the mutation oligonucleotide 5'-CTGCGTGTT GCTCCGGTCAACTTCAAACTG-3' (SEQ ID NO: 2, mutated codon α D94A is underlined) and 5'-GGAAAACTTCCGA TGGCTGGGTAACGTAC-3' (SEQ ID NO: 3, mutated codon β L105W is underlined) were used. Both oligonucleotides were purchased from DNA International, Inc. (Lake Oswego, Oregon).

The construction of plasmid pHE2017 (mutant rHb (α D94A, β L105W)) was accomplished by ligating the 0.51-kb SmaI-PstI fragment of pHE2020 with the 6.34-kb PstI-SmaI fragment of pHE2004. The construction of plasmid pHE2018 for

expression of mutant rHb (α L29F, β N108Q) was accomplished by ligating the 6.06-kb PstI-BamHI fragment of pHE2009 with the 0.79-kb BamHI-PstI fragment of pHE284. The construction of plasmid pHE284 containing the mutation α L29F from plasmid pHE2 was reported previously by Jeong, et al. (1999).

5 The construction of plasmid pHE7009 for expression of mutant rHb (β N108Q) using the human globin cDNAs was carried out as follows. The coding sequences of human α - and β -globin cDNAs in plasmid pHE7 were inserted into pTZ18U (Bio-Rad Laboratories, Hercules, CA) by methods well known in the art. Site-directed mutagenesis was performed as described by Kunkel, T.M. et al., Proc. Natl. Acad. Sci. USA 82:488 (1985) the disclosures of which are incorporated herein by
10 reference, and Shen, et al. (1993). An oligonucleotide of sequence 5'-ACAGACCAG TACTTGTCC CAGGAGCCT-3' (SEQ ID NO: 4) (mutated codon Asn→Gln is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon), and used as the mutation primer. The human normal β -globin cDNA in plasmid pHE7 was
15 then replaced with the mutated cDNA to produce plasmid pHE7009. The DNA sequences for the α - and β -globin cDNAs in pHE7009 are shown in Figure 1A (SEQ ID NO: 5). Plasmid pHE7009 in host cell E. coli JM109 and designated pHE7009/JM109 was deposited with the American Type Culture Collection of Manassas, VA on April 27, 2000 under number PTA-1768.

20 The construction of plasmid pHE7004 for expression of mutant rHb (β L105W) using the human globin cDNAs was carried out in the similar way as that of plasmid pHE7009, except an oligonucleotide of sequence 5'-CCTGAGAACTTC

AGGTGGCTAGGCAACGTGCTGGTC-3' ((SEQ ID NO: 6), mutated codon Leu→Trp is underlined) was purchased from DNA International, Inc. (Lake Oswego, Oregon) and used as the mutation primer. The DNA sequences of the α - and β -globin cDNAs in pHE7004 are shown in Figure 1B (SEQ ID NO: 7). Plasmid pHE7004 in host cell E. coli JM109 and designated pHE7004/JM109 was deposited with the American Type Culture Collection of Manassas, VA on April 27, 2000 under number PTA-1769.

Growth of Cells

Plasmids pHE7009, and pHE7004 were individually transformed in E. coli strain JM109 (Promega, Madison, WI) by methods well known in the art. E. coli cells were grown in Terrific Broth ("TB") medium plus 100 μ g/mL ampicillin in a 10-liter Microferm fermenter (New Brunswick Scientific, Model BioFlo 3000) at 32°C until the optical density at 600 nm reached 10. TB medium contains 1.2% bactotryptone, 2.4% bacto-yeast extract, 0.17M KH_2PO_4 , 0.072M K_2HPO_4 , and 1% glucose solution. Expression of rHbs was induced by adding isopropyl β -thiogalactopyranoside (Sigma, St. Louis, MO) to a concentration of 0.1-0.4 mM. The culture was then supplemented with hemin (20-50 mg/liter) (Sigma) and the growth was continued for at least another 4 hr. The cells were harvested by centrifugation and stored frozen at -80°C until needed for purification. For details, refer to Shen, et al. (1993), and Shen, et al. (1997).

Although E. coli cells are presently preferred for expressing and producing the recombinant mutant hemoglobin of the present invention, the invention is not limited to E. coli cells. Other appropriate expression systems such as yeast, insect cells and transgenic animals such as pigs, sheep, and cows may also advantageously be

used to express mutant hemoglobins. Plasmids pHE7009 and pHE7004 have been optimized for *E. coli* cells, but other expression systems may be advantageously used. The plasmids can also be constructed with human genes.

Isolation and Purification of rHbs

5 The recombinant hemoglobins obtained from cells transformed with plasmids pHE7009 and pHE7004 were purified as essentially described by Shen, et al. (1993), and Shen, et al. (1997). The frozen stored cell paste was put into lysis buffer (40 mM trisbase/1 mM benzamidine) (Sigma) at 3 ml/gm of cell paste). The cell lysis procedure was used to pass the cell paste through a high-pressure homogenizer (Model 10 EmulsiFlex-C5, Avestin) 3 times. The lysate was then centrifuged at 4°C for 2.5 hours at 13,000 rpm in a Beckman centrifuge (Beckman JA14 rotor). The supernatant from the lysate was saturated with CO gas and left at 30°C overnight as described by Tsai, et al. (1999). The supernatant was then put through a Millipore Minitan Acrylic Ultrafiltration system to concentrate the protein. Polyethyleneimine (Sigma) was added 15 to a final concentration of 0.5% to precipitate nucleic acids. After centrifugation, the sample was dialyzed against 20 mM Tris-HCl/0.5 mM triethylenetetraamine ("TETA") (Sigma) at pH 8.3 overnight with one or two changes of buffer. Throughout the above procedures, the sample was kept at 4°C and maintained in a CO atmosphere. Following the procedures of Shen, et al. (1993) and Shen, et al. (1997), the rHb fraction 20 collected after passage through a Q-Sepharose Fast-Flow column (Pharmacia Anion Exchanger) Pharmacia was oxidized and reduced, and converted to the CO form. This Hb solution was then purified by eluting through a fast protein liquid chromatography

("FPLC") Mono-S column (Pharmacia Cation Exchanger, HR 16/10) with a gradient of 10 mM sodium phosphate in 0.1-0.5 mM ethylenediaminetetraacetic acid ("EDTA") at pH 6.8 (eluent A) and 20 mM sodium phosphate in 0.1-0.5 mM EDTA at pH 8.3 (eluent B).

5 rHb (β N108Q) was eluted individually in two major peaks. Figure 2A shows peak a and peak b for rHb (β N108Q). Figure 2B shows rHb (β L105W) was eluted individually in three major peaks, peaks a, b, and c. rHbs collected from peak b in both cases contained less than 2% methionine at the amino-termini and with the correct molecular weight.

10 Mass Spectrometry

Hb samples subjected to mass spectrometry were dialyzed extensively against distilled H₂O and then lyophilized. Immediately before analysis, the samples were dissolved in water to a concentration of 125 pmol of Hb per μ l of H₂O (7.8 mg/ml). Aliquots of these solutions were then diluted to give a final concentration of 15 10 pmol/ μ l of 50:50 water/acetonitrile containing 0.2% formic acid. Aliquots (10 μ l) of these final solutions were introduced into the electrospray ion source at 5 μ l/minute.

The electrospray ionization analyses were performed on a VG Quattro-BQ (Fisons Instruments, VG Biotech, Altrincham, U.K.), as described by Shen, et al. (1993).

Automated cycles of Edman degradation were performed on an Applied Biosystems 20 gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A). These two analytical procedures were used to assess the quality of the rHbs. All rHbs used in this study had

the correct molecular weights and contained less than 2% of methionine at the amino termini.

Oxygen-Binding Properties of rHbs

Oxygen dissociation curves of rHbs were measured by a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29°C as a function of pH. The concentration of Hb used was approximately 0.1 mM per heme. The methemoglobin ("met-Hb") reductase system described by Hayashi, A., et al. Biochem. Biophys. Acta 310:309 (1973), the disclosure of which is incorporated herein by reference, was used if needed to reduce the amount of met-Hb in the sample. A visible absorption spectrum of each sample was recorded immediately after oxygen equilibrium measurement, and the met-Hb content was estimated by using the extinction coefficients of Hb reported by Antonini, E., Physiol. Rev. 45:123 (1965), the disclosure of which is incorporated herein by reference. Oxygen equilibrium parameters were derived by fitting Adair equations to each equilibrium oxygen-binding curve by a nonlinear least-squares procedures. P_{50} , a measure of oxygen affinity, was obtained at 50% saturation. The Hill coefficient (n_{\max}), a measurement of cooperativity, was determined from the maximum slope of the Hill plot by linear regression. n_{\max} was derived between 60% and 65% oxygen saturation. The accuracy of P_{50} measurements in mm Hg is $\pm 5\%$ and that for n_{\max} is $\pm 7\%$.

^1H -NMR Spectroscopy Measurements of rHbs

^1H -NMR spectra of rHbs were obtained on Bruker AVANCE DRX-300, AVANCE DRX-500, and AVANCE DRX-600 NMR spectrometers that were operated at

300, 500, and 600 MHz, respectively, and at temperatures ranging from 10° C-36° C. All of the Hb samples were placed in 0.1 M sodium phosphate buffer (in 100% H₂O) at pH 7.0. The Hb concentration range was approximately 5% (~3 mM in terms of heme). The water signal was suppressed by using the "jump-and-return" pulse sequence as reported by Plateau, P., et al. J. Am. Chem. Soc. 104:7310 (1982) (hereinafter "Plateau, et al. (1982)"), the disclosure of which is incorporated herein by reference. Proton chemical shifts are referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5 sulfonate ("DSS") indirectly by using the water signal, which signal occurs at 4.76 ppm downfield from that of DSS at 29°C, as the internal reference.

Autoxidation of rHbs

The autoxidation of rHbs was recorded by monitoring the disappearing rate of the oxy-marker (-2.34 ppm from DSS) from Bruker AVANCE DRX-300 ¹H-NMR spectra. The autoxidation reaction was carried out in PlasmaLyte buffer (Baxter) (5% D₂O) with 5 mM EDTA at pH 7.4 and at 37°C. The HbO₂ concentration was 5% (~3 mM in terms of heme).

FUNCTIONAL STUDIES

Oxygen-binding Properties of rHbs

Figures 3A and 3B show the oxygen-binding measurements of rHb (αL29F), rHb (βN108Q), rHb (α29F, βN108Q), rHb (βLI05W), and Hb A in 0.1 M sodium phosphate buffer as a function of pH at 29°C. rHb (βN108Q) exhibits a significantly lower oxygen affinity as compared to that of Hb A over the pH range from pH 6.79 to pH 8.09. The oxygenation process of rHb (βN108Q) is very cooperative with an n_{max}

value of about 2.7 to 3.1 depending on the pH, compared to about 3.2 for Hb A (Figure 3B). On the other hand, the mutation at the α -chain B10 position, i.e., α L29F, increases the oxygen affinity and decreases the cooperativity. rHb (α L29F, β N108Q) shows slightly higher P_{50} values as compared to those of Hb A at pH < 7.4, suggesting that the effect of the mutations on the oxygen affinity is additive. rHb (α L29F, β N108Q) preserves cooperativity in binding of oxygen with an n_{\max} value of 2.4 to 2.8 (Figure 3B). rHb (β L105W) exhibits very low oxygen affinity (about 2-3 times lower) and maintains normal cooperativity from pH 7.0 to 8.0 as compared to Hb A.

Figure 4 shows that the oxygen affinities of rHb (β N108Q) and also rHb (β L105W) measured in the absence of 2,3-BPG are lower than that of Hb A in the presence of 5 mM 2,3-BPG, making them potential candidates for an oxygen carrier in a blood substitute system. Figures 3A and 3B also show that the alkaline Bohr effect (which, in Hb A, results in a decrease in oxygen affinity with a lowering of the pH) is enhanced in rHb (β N108Q) and rHb (α L29F, β N108Q) compared to Hb A.

Table 1 below compares the number of Bohr protons released upon oxygenation per heme calculated from the linkage equation $\Delta H^+ = -\partial \log P_{50} / \partial \text{pH}$. (Wyman, J., Adv. Protein Chem. 4:407 (1948) and Adv. Protein Chem. 19:233 (1964), (hereinafter "Wyman, J. (1948) and (1964)") the disclosures of which are incorporated herein by reference. Both rHb (β N108Q) and rHb (α L29F, β N108Q) release more Bohr protons than Hb A.

Table 1

Bohr effect of Hb A, rHb (β N108Q), rHb (α L29F, β N108Q), and rHb (β L105W)
in 0.1 M sodium phosphate buffer at 29°C

<u>Hemoglobin</u>	<u>$\partial \log P_{50} / \partial \text{pH}$ in 0.1 phosphate</u>
Hb A	0.48 (pH 6.79-8.00)
rHb (β N108Q)	0.56 (pH 6.79-8.09)
rHb (α L29F, β N108Q)	0.67 PpH 6.79-7.97)
rHb (β L105W)	0.55 (pH 7.00-8.00)

Autoxidation.

The autoxidation process was monitored for oxy-Hb A, oxy-rHb (β N108Q), oxy-rHb (α L29F, β N108Q), oxy-rHb (β L105W) and three other known low-oxygen affinity mutants, oxy-rHb (α V96W), oxy-rHb (α V96W, β N108K) and oxy-rHb (α L29F, α V96W, β N108K), by a 300-MHz NMR spectrometer. The resonance at -2.34 ppm upfield from DSS has been assigned to the γ_2 -CH₃ of E11Val of Hb A in the oxy form of Hb A (Dalvit, C., et al., Biochemistry 24:3398 (1985), the disclosure of which is incorporated herein by reference). Monitoring the rate of disappearance of the oxy-marker (-2.34 ppm from DSS) as a function of time allows for the determination of the autoxidation rate of the Hb samples. The results are shown in Figure 5. The percentage of ferrous-Hb varies with time (t) mono-exponentially and the autoxidation rate constant can be obtained from: $[\text{ferrous-Hb}]_t = [\text{ferrous-b}]_{t=0} \times \exp(-k_{\text{auto}} \times t)$, where k_{auto} is the autoxidation rate constant. The autoxidation rate constants of Hb A and r Hbs are

listed in Table 2 below. At pH 7.4 and 37°C in PlasmaLyte buffer, rHb (β N108Q), rHb (β L105W), rHb (α V96W), and rHb (α V96W, β N108K) autoxidized 2.8-, 8-, 4.4-, and 8-times faster than Hb A. rHb (β N108Q) is shown to be more stable against autoxidation than other known low-oxygen affinity mutants developed in the laboratory; i.e., rHb (α V96W), rHb (β L105W), and rHb (α V96W, β N108K). The autoxidation rate is slowed down by introducing the mutation α L29F into rHb (β N108Q) and rHb (α V96W, β N108K). rHb (α L29F, β N108Q) and rHb (α L29F, α V96W, β N108K) autoxidized 2.5- and 2.8-times slower than rHb (β N108Q) and rHb (α V96W, β N108K), respectively. Thus, the mutation α L29F is very effective in slowing down the autoxidation process as suggested by the results on myoglobin (Brantley, et al., (1993), the disclosure of which is incorporated herein by reference). Hemichrome-like spectra are observed in the autoxidation process of only rHb (α L29F, α V96W, β N108K) among all the low oxygen affinity rHbs studied. Hemichrome forms when methemoglobin (met-Hb) converts from the ferric high-spin form to the ferric low-spin form in which the distal imidazole displaces the H₂O ligand (Levy et al., Biochemistry 29: 9311 (1990); Levy, et al., Biophys. J. 61: 750 (1992); Blumberg, et al., Adv. Chem. Series 100: 271 (1991)). This is in accordance with the results from Jeong et al. (1999) in which the oxidized form of rHb (α L29F, α V96W, β N108K) exhibits hemichrome-like spectra, making it undesirable to be considered as a candidate for an oxygen carrier.

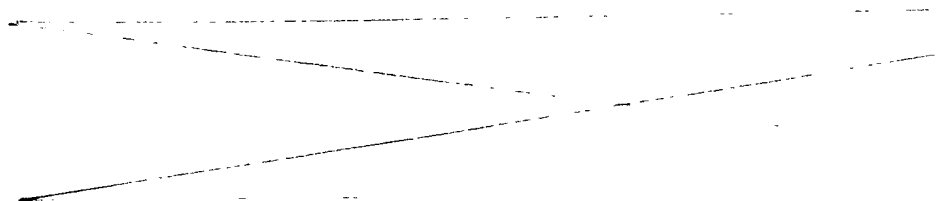


Table 2

Autoxidation rate constants, oxygen affinity and cooperativity
of low-oxygen affinity mutants.

<u>Hemoglobin</u>	<u>k_{auto} (s^{-1})^a</u>	<u>P_{50} (mm Hg)^b</u>	<u>n_{max}^c</u>
Hb A	0.0158 ± 0.0002	9.64	3.28
rHb(β N108Q)	0.0449 ± 0.0007	17.46	3.10
rHb (α L29F, β N108Q)	0.0181 ± 0.0006	12.06	2.77
rHb (β L105W)	0.123 ± 0.0048	28.2	2.60
rHb (α V96W)	0.0689 ± 0.0008	16.38	2.94
rHb (α V96W, β N108K)	0.125 ± 0.0051	50.65	2.36
rHb (α L29F, α V96W, β N108K)	0.0449 ± 0.0014	21.97 ^c	1.81 ^c

a Rate constants for the autoxidation (k_{auto}) of Hb A and r Hbs are obtained at [oxy-Hb] 3 mM heme in PlasmaLyte buffer at pH 7.4 and 37°C.

b Oxygen affinity and cooperativity were obtained in 0.1 M sodium phosphate buffer at 29°C and pH 7.4. Protein concentrations were 0.1 mM heme.

c From Jeong, et al. (1999).

STRUCTURAL STUDIES OF rHb (β N108Q) AND rHb (α L29F, β N108K)

^1H -NMR Investigations

^1H -NMR spectroscopy is an excellent tool for monitoring changes in the tertiary and quaternary structures of Hb A and its variants (see, e.g., Shen, et al. (1993); Kim, et al. (1994); Kim, et al. (1995); Kim, et al. (1996); and Barrick, D., et al. Nat. Struct. Biol. 4:78 (1997), the disclosures of which are incorporated herein by reference). Figure 6A shows the exchangeable proton resonances and Figure 6B shows the ring-current-

shifted resonances of Hb A, rHb (β N108Q), and rHb (α L29F, β N108Q) in the CO form measured at 500 MHz. The ring-current-shifted resonances are sensitive to the orientation and/or conformation of the heme group relative to the amino acid residues in the heme pockets, i.e., the tertiary structure of the Hb molecule (see, Ho, C. , Adv.

5 Protein Chem. 43:153 (1992), (hereinafter "Ho (1992)"), the disclosure of which is incorporated herein by reference). The resonances at ≈ -1.8 and ≈ -1.7 ppm have been assigned to the γ_2 -CH₃ of the E11Val of the β -chain and α -chain of HbCO A, respectively (Lindstrom et al. (1972); Dalvit et al. (1985)). These two resonances are not changed in rHbCO (β N108Q). However, the resonance assigned to the γ_2 -CH₃ of the α -E11Val of
10 rHbCO (α L29F, β N108Q) is shifted upfield to ≈ -2.0 ppm, suggesting that the γ_2 -CH₃ group of the α -E11 valine residues in rHbCO (α L29F, β N108Q) is located closer to the normal of the heme than in HbCO A. α 29L is in close proximity to E11Val, hence, the amino acid substitution α L29F is expected to alter the conformation of the distal heme pocket site of the α -chain. There are some other changes in the ring-current-shifted
15 resonances among these rHbs. The experience has been that minor differences in the intensity and positions of ring-current-shifted resonances are common features in many rHb mutants. (See, for example, Shen, et al. (1993); Kim, et al. (1994); Kim, et al. (1995); and Kim, et al. (1996); Ho, et al. (1998); Sun, D.P., et al. Biochemistry 36:6663 (1997) (hereinafter "Sun, et al. (1997)"), the disclosure of which is incorporated herein by
20 reference; and Tsai, et al. (1999)). These changes reflect slight adjustments of the conformation of the hemes and/or the amino acid residues in the heme pockets as the result of the mutation.

5 The exchangeable proton resonances of the Hb molecule arise from the exchangeable protons in the subunit interfaces. Of special interest to the present invention are the exchangeable proton resonances at 14.2, 12.9, 12.1, 11.2, and 10.7 ppm from DSS, which have been characterized as the inter-subunit H-bonds in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in both deoxy (T) and/or oxy (R) states of Hb A (Russu, et al (1987); Fung, et al. (1975)); and Ho (1992), the disclosures of which are incorporated herein by reference). The resonances at 12.9 ppm and 12.1 ppm from DSS have been assigned to the H-bonds between $\alpha_{122}\text{His}$ and $\beta_{35}\text{Tyr}$, and $\alpha_{103}\text{His}$ and $\beta_{131}\text{Gln}$, respectively (see Russu, et al. (1987) and Simplaceanu, et al. Biophys. J. (in press) (2000) (hereinafter "Simplaceanu, et al. (2000)"). In the spectra of rHbCO (βN108Q) and rHbCO (αL29F , βN108Q) (as seen in Figure 6A), three resonances instead of one occur corresponding to the chemical shift of HbCO A at 12.1 ppm. The main peak occurs at 12.0 ppm, with a shoulder at 11.8 ppm and an extra resonance at 12.3 ppm. The intensities of the resonances at 12.3 and 11.8 ppm are not even 1/10 of the ones at 12.0 ppm and at 12.9 ppm, indicating that these two extra resonances are unlikely to be contributed by additional protons. The sum of the integrated areas of the resonances at 11.8, 12.0, and 12.3 ppm is about the same as the area of the single resonance at 12.9 ppm, suggesting the coexistence of three conformers of rHb (βN108Q) in CO form.

Figure 7A shows the hyperfine-shifted and Figure 7B shows the exchangeable proton resonances of rHbs and Hb A in the deoxy form in 0.1 M phosphate at pH 7.0 and 29°C. The resonance at 63 ppm from DDS has been assigned to the hyperfine-shifted N_δH -exchangeable proton of the proximal histidine residue

(α 87His) of the α -chain of deoxy-Hb A and the one at 77 ppm from DSS has been assigned to the corresponding residue of the β -chain (β 92His) of deoxy-Hb A (Takahashi, S., et al. Biochemistry 19:5196 (1980) and La Mar, G.N., et al. Biochem. Biophys. Res. Commun. 96:1172 (1980), the disclosures of which are incorporated herein by reference). The chemical shift positions of these two proximal histidyl resonances in rHb (β N108Q) are exactly the same as those of Hb A, indicating no perturbations around the proximal histidine residues of this rHb. However, the resonance at 63 ppm from DSS of rHb (α L29F) and rHb (α L29F, β N108Q) is shifted 4 ppm downfield to 67 ppm, reflecting a change in the environment of the proximal heme pocket of the α -chain as a result of the mutation at α L29F.

The spectral region from 10-25 ppm arises from the hyperfine-shifted resonances of the porphyrin ring and the amino acid residues situated in the proximity of the heme pockets and the exchangeable proton resonances. There are no noticeable differences seen in the resonances from 10-25 ppm between deoxy-Hb A and deoxy-rHb (β N108Q). However, there are spectral changes in rHb (α L29F) and rHb (α L29F, β N108Q) over the region from 16-20 ppm, reflecting changes in the environment of the heme pockets of both the α - and the β - chains. The resonance at 14.2 ppm has been identified as the inter-subunit H-bond between α 42Tyr and β 99Asp in the $\alpha_1\beta_2$ interface in deoxy-Hb A (Fung, et al. (1975)), a characteristic feature of the deoxy (T) quaternary structure of Hb A (Perutz, (1970)). This resonance of rHb (α L29F) and rHb (α L29F,

βN108Q) is shifted 0.5 ppm upfield to 13.7 ppm, indicating that this α₁β₂ interface H-bond in the deoxy form is perturbed by the mutation at αL29F.

A unique feature of the rHbs of the present invention with low oxygen affinity and high cooperativity is the appearance of the T-marker at 14.2 ppm on lowering the temperature and/or adding IHP to these rHbs in the CO form (see Kim, et al. (1995); Ho, et al. (1998); Tsai, et al. (1999)). Studies on the temperature dependence of exchangeable proton resonances of rHbs in the CO form can be used to assess the structural effect on oxygen affinity. Figures 8A and 8B and Figures 9A and 9B show the exchangeable proton resonances of rHb (βN108Q) and rHb (αL29F, βN108Q) in the CO form in the absence (Figs. 8A, 9A) and presence (Figs. 8B, 9B) of 4 mM IHP in 0.1 M sodium phosphate buffer as a function of temperature. The resonance at 14.2 ppm of rHb (βN108Q) is observable starting at 23°C in 0.1 M phosphate at pH 7.0 in the presence of 4 mM IHP (Figure 8B). The appearance of the T-marker in the presence of 4 mM IHP and at low temperature in the spectra of CO-ligated rHb (βN108Q) and rHb (αL29F, βN108Q) indicates that the T-states of rHb (βN108Q) and rHb (αL29F, βN108Q) are more stable than that of Hb A. However, this resonance in the spectra of Hb (αL29F, βN108Q) has a much smaller intensity than that in the spectra of rHb (βN108Q) at low temperature, i.e., 11°C and in the presence of 4 mM IHP.

STRUCTURAL STUDIES OF rHb (βL105W)

rHb (βL105W) was designed to form a new hydrogen bond with α94Asp in the α₁β₂ subunit interface in order to lower the oxygen binding affinity by stabilizing its deoxy quaternary structure. rHb (αD94A, βL105W) and rHb (αD94A) were

constructed to provide evidence that $\beta 105\text{Trp}$ of rHb (βL105W) does form a new hydrogen bond with $\alpha 94\text{Asp}$ in the $\alpha_1\beta_2$ subunit interface of the deoxy quaternary structure. Multinuclear, multidimensional nuclear magnetic resonance (NMR) studies on ^{15}N -labeled rHb (βL105W) have identified the indole nitrogen-attached proton resonance of $\beta 105\text{Trp}$ for rHb (βL105W). ^1H -NMR studies were used to investigate the structural basis for the low oxygen affinity of rHb (βL105W).

^1H -NMR Studies of the Structures of rHbs in the CO Form.

Figure 10A shows the exchangeable proton resonances of Hb A, rHb (βL105W), rHb (αD94A , βL105W), and rHb (αD94A) in the CO form. The exchangeable proton resonances arise from the exchangeable protons in the subunit interfaces. Recent multinuclear, multidimensional NMR studies on the ^{15}N -labeled rHb A have assigned the resonances at 10.6, 10.4 and 10.1 ppm to $\beta 37\text{Trp}$, $\alpha 14\text{TIP}$ and $\beta 15\text{Trp}$, respectively (Simplaceanu, et al. (2000)). The crystal structure of Hb A in the oxy form (Shaanan, (1983)) suggested the likely candidate to form an H-bond with $\beta 37\text{Trp}$ in the $\alpha_1\beta_2$ subunit interface is $\alpha 94\text{Asp}$. The spectrum of rHb (βL105W) in the CO form shows an additional proton resonance in the region of exchangeable proton resonances (Figure 10A). Since residues $\beta 37$ and $\beta 105$ are both located in the $\alpha_1\beta_2$ interface and are close in the R-quaternary structure (Shaanan, (1983)), the replacement of Leu by Trp at $\beta 105$ position may cause the proton resonance of $\beta 37\text{Trp}$ to shift away from its original chemical shift. It is suspected that the extra resonance (at either 11.0 ppm or 10.8 ppm) originates from $\beta 105\text{Trp}$. Heteronuclear, two-dimensional ("2D") NMR studies on the

¹⁵N-labeled rHb (βL105W) were, therefore, carried out to assign these resonances in the spectrum of rHb (βL105W). The spectrum of rHb (αD94A) in the CO form shows that the resonance at 10.6 ppm (assigned to β37Trp in Hb A) is missing and a new resonance shows up at 9.7 ppm compared to the spectrum of Hb A (Figure 10A). This result suggests that the shift of the resonance of β37Trp at 10.6 ppm to 9.7 ppm (closer to the water resonance) is due to the lack of an H-bond between α94 and β37 in rHb (αD94A) in the CO form. This result also confirms the assignment of the resonance at 10.6 ppm to the inter-subunit H-bond between α94Asp and β37Trp. The spectrum of rHb (αD94A, βL105W) in the CO form shows that one extra proton resonance appeared at 10.8 ppm compared to the spectrum of rHb (αD94A). The resonance at 10.8 ppm was assigned to the indole NH of β105Trp of rHb (αD94A, βL105W) and rHb (βL105W).

Figure 10B shows the ring-current-shifted proton resonances of Hb A, rHb (βL105W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form. The ring-current-shifted resonances are very sensitive to the heme conformation and the tertiary structure of the heme pockets (Ho, (1992)). The spectrum for the ring-current-shifted proton resonances of rHb (βL105W) in the CO form differs only slightly from that of Hb A, while the spectra of rHb (αD94A, βL105W) and rHb (αD94A) are very different from that of Hb A. These differences imply that some adjustments of the heme conformation and/or the amino acid residues in the heme pockets occurred due to the mutation αD94A. Previous studies have shown that minor differences in the ring-current-shifted

resonances are common features in many mutant rHbs. (Kim, et al. (1994); Kim, et al. (1995); Kim, et al. (1996); Sun, et al. (1997)).

Heteronuclear 2D NMR Studies on ^{15}N -labeled rHb (βL105W) in the CO Form.

In order to assign the proton resonances at 11.0 ppm and 10.8 ppm in the ^1H -NMR spectrum of rHb (βL105W), heteronuclear 2D NMR studies on ^{15}N -labeled rHb (βL105W) in the CO form were performed. The results are shown in Figures 11A and 11B and Figures 12A-12D. Figures 11A and 11B show the 600-MHz HMQC spectra of ^{15}N -labeled rHb (βL105W) and rHb A in the CO form. A doublet is observed at the ($^1\text{H}\epsilon_1$, $^{15}\text{N}\epsilon_1$) chemical shift coordinates for Trp residues because this spectrum was acquired without ^{15}N decoupling. In general, the $^1\text{H}\epsilon_1$ resonances of Trp residues usually appear at ~ 9 to ~ 12 ppm (Cavanagh, et al. (1996); BioMagResBank (1999) (www.bmrb.wisc.edu/ref_info/statsel.htm) in the proton dimension, and their $^{15}\text{N}\epsilon_1$ resonances usually appear at ~ 121 to ~ 133 ppm (BioMagResBank) in the ^{15}N dimension. The ^{15}N chemical shifts for the proton resonances at 11.0 ppm and 10.8 ppm in the ^1H -NMR spectrum of rHb (βL105W) are at 134 ppm and 129 ppm, respectively, suggesting that these resonances originate from a Trp residue. Since the chemical shift of a proton is much easier to be affected than that of nitrogen by its environment, the assignment of (11.0, 134) ppm to $\beta 37\text{Trp}$ and (10.8, 129) ppm to $\beta 105\text{Trp}$ was made (Figures 11A and 11B). This also agrees with what is shown in Figure 10A. The HMQC spectrum also correlates the Trp $^{15}\text{N}\epsilon_1$ chemical shifts with the carbon-bound proton $^1\text{H}\delta_1$. As shown in Figures 11A and 11B, the $^1\text{H}\delta_1$ cross-peaks at (7.3, 129) and (7.1, 127) ppm are observed for $\alpha 14\text{Trp}$ and $\beta 15\text{Trp}$, respectively, in the spectra of both ^{15}N -

labeled rHb (β L105W) and rHb A in the CO form. Also observed are $^1\text{H}\delta_1$ cross-peaks (through two-bond coupling) at (7.4, 134) ppm with much weaker intensity for β 37Trp in the spectrum of ^{15}N -labeled rHb A in the CO form (results not shown in Figure 11B). Since the $^1\text{H}\delta_1$ cross-peaks for β 37Trp and β 105Trp cannot be seen in the spectrum of ^{15}N -labeled rHb (β L105W) in the CO form (Figure 11A), NOESY-HMQC experiments have been performed at different mixing times to provide more evidence for the present Trp assignments. As shown in Figures 12A-12D, the $^1\text{H}\delta_1$ and $^1\text{H}\zeta_2$ cross-peaks of all four Trp residues can be seen even at short mixing times. The intensities of these cross-peaks become weaker when the mixing time was 60 or 100 ms (Figs. 12C, 12D). Figures 12A-12D also show that the chemical shifts of $^1\text{H}\delta_1$ and $^1\text{H}\zeta_2$ cross-peaks are very close to each other for β 37Trp and β 105Trp. All these results confirm the assignments for the Trp residues.

^1H -NMR Studies of the Structures of rHbs in the Deoxy Form.

Figure 13A shows the hyperfine-shifted N_δH resonances of proximal histidines in the 300-MHz ^1H -NMR spectra of Hb A, rHb (β L105W), rHb (α D94A, β L105W), and rHb (α D94A) in the deoxy form. The spectrum for the hyperfine-shifted N_δH resonances of proximal histidines of mutant rHbs in the deoxy form is very similar to that of Hb A. Figure 13B shows the hyperfine-shifted and exchangeable proton resonances in the 300-MHz ^1H -NMR spectra of Hb A, rHb (β L105W), rHb (α D94A, β L105W), and rHb (α D94A) in the deoxy form. The hyperfine-shifted proton resonances arise from the protons on the heme groups and their nearby amino acid

residues due to the hyperfine, interactions between these protons and unpaired electrons of Fe(II) in the heme pocket (Ho (1992)). The hyperfine-shifted proton resonances of rHb (β L105W) in the region +24 to +16 ppm are very similar to those of Hb A, but those for rHb (α D94A, β L105W) and rHb (α D94A) are somewhat different from these for Hb A. Figure 13C shows the exchangeable proton resonances in the 300-MHz ^1H -NMR spectra of Hb A, rHb (β L105W), rHb (α D94A, β L105W), and rHb (α D94A) in the deoxy form. The ^1H resonance at ~ 14 ppm has been identified as the intersubunit H-bond between $\alpha 42\text{Tyr}$ and $\beta 99\text{Asp}$ in the $\alpha_1\beta_2$ interface in deoxy-Hb A, a characteristic feature of the deoxy (T)-quaternary structure of Hb A (Fung, et al. (1975)).

The resonance at ~ 12.2 ppm has been assigned to the H-bond between $\alpha 103\text{His}$ and $\beta 131\text{Asp}$ at the $\alpha_1\beta_2$ interface (unpublished results). The resonance at ~ 11.1 ppm has been tentatively assigned to the H-bond between $\alpha 94\text{Asp}$ and $\beta 37\text{Trp}$ at the $\alpha_1\beta_2$ interface (Fung, et al. (1975); Ishimori, et al. (1992)). Recent heteronuclear, multidimensional NMR studies on the ^{15}N -labeled rHb A have assigned the resonance at ~ 13.0 ppm to $\alpha 122\text{His}$, and confirmed the assignment of the resonance at ~ 11.1 ppm to $\beta 37\text{Trp}$ (unpublished results). The spectrum of rHb (β L105W) in the deoxy form shows an additional proton resonance appearing in the region of the exchangeable proton resonances (Figure 13C). This suggests that the extra resonance at 12.7 ppm originates from $\beta 105\text{Trp}$. Due to the lack of an H-bond between residues $\alpha 94$ and $\beta 37$ in rHb (α D94A) in the deoxy form, the resonance for $\beta 37\text{Trp}$ should shift away from its original chemical shift and closer to the water resonance (similar to what was observed

in its CO form). The spectrum of rHb (α D94A) in the deoxy form shows that the resonance at ~ 11.1 ppm (assigned to β 37Trp in Hb A) is missing (Figure 13C).

However, it is not clear what is the new chemical shift for β 37Trp in rHb (α D94A) in the deoxy form. The spectrum of rHb (α D94A, β L105W) in the deoxy form shows an extra proton resonance appearing at 11.1 ppm compared to that of rHb (α D94A). It appears that this resonance originates from β 105Trp of rHb (α D94A, β L105W). The chemical shift for the NH resonance of β 105Trp in rHb (β L105W) shifts upfield 1.7 ppm and closer to the water resonance when α 94Asp is replaced by Ala in rHb (α D94A, β L105W) (Figure 13C). These results indicate that a new H-bond forms between β 105Trp and α 94Asp in rHb (β L105W) in the deoxy form.

Heteronuclear 2D NMR Studies on ^{15}N -labeled rHb (β L105W) in the Deoxy Form.

In order to confirm the assignment of resonance at 12.7 ppm to β 105Trp in the ^1H -NMR spectrum of rHb (β L105W) in the deoxy form, heteronuclear 2D NMR experiments on ^{15}N -labeled rHb (β L105W) in the deoxy form were performed (Figures 14 and 15A-15D). Figure 14 shows the 600-MHz HMQC spectrum of ^{15}N -labeled rHb (β L105W) in the deoxy form. The ^{15}N chemical shift for the proton resonance at 12.7 ppm in the ^1H -NMR spectrum of rHb (β L105W) is at 134 ppm, suggesting that this resonance originates from a tryptophan residue. Also observed are the $^1\text{H}\delta_1$ cross-peaks of Trp $^{15}\text{N}\epsilon_1$ at (7.8, 134), (7.6, 135), (7.1, 129), and (7.0, 127) ppm for β 105Trp, β 37Trp, α 14Trp and β 15Trp, respectively, in the HMQC spectrum of ^{15}N -labeled rHb (β L105W) in the deoxy form. Also observed are the $^1\text{H}\epsilon_1$ and $^1\text{H}\zeta_2$ cross-peaks of His $^{15}\text{N}\epsilon_2$ for

α103His at (8.3, 163) and (7.1, 163) ppm, respectively, and for α122His at (7.6, 167) and (7.0, 167) ppm, respectively. The NOESY-HMQC experiments were also performed at different mixing times to provide more evidence for the present assignments and to investigate the micro-environment for β105Trp in rHb (βL105W) in the deoxy form. For β105Trp (at 12.7 ppm), its ¹Hδ₁ and ¹Hζ₂ cross-peaks at 7.8 and 8.2 ppm, respectively, can be observed at all four mixing times (Figures 15A-15D). For β37Trp (at 11.2 ppm), its ¹Hδ₁ and ¹Hζ₂ cross-peaks at 7.6 and 7.3 ppm, respectively, also can be observed at all four mixing times (Figures 15A-15D). In addition, also observed is the NOE effect between residues of β105Trp and β37Trp in the NOESY-HMQC spectra of ¹⁵N-labeled rHb (βL105 W) in the deoxy form as shown in Figures 15A-15D.

The Effects of IHP and Temperature on the Spectra of Hbs in the CO Form

Figures 16A and 16B show the exchangeable protons resonances of Hb A, rHb (βL105W), rHb (αD94A, βL105W), and rHb (αD94A) in the CO form in the absence (Fig. 16A) and presence (Fig. 16B) of IHP at 11, 20, and 29°C. In the absence of IHP, the T marker can be observed only in the spectra of rHb (αD94A, βL105 W) at the lower temperature. In the presence of IHP, the T marker can be observed in the spectra of all three mutant rHbs. These results have shown that these mutant rHbs can switch from the R-structure to the T-structure without changing their ligation state when the temperature is lowered and/or when IHP is added. Besides the appearance of the T marker, the spectra of rHb (βL105W) in the CO form in the presence of IHP also show several differences compared to those in the absence of IHP. In the presence of IHP, new peaks at 13.1 and 11.2 ppm appear to gradually grow from the nearby resonances

at 12.9 and 11.0 (or 10.8) ppm, respectively, when the temperature is lowered (Figure 16B).

In order to monitor these changes, more detailed HSQC experiments have been performed for ^{15}N -labeled rHb (βL105W) in the absence and the presence of IHP.

5 In the presence of IHP, the HSQC experiments have also been performed at lower temperatures (Figures 17A-17D). In the presence of IHP at 29°C , the ($^1\text{H}_{\epsilon 1}$, $^{15}\text{N}_{\epsilon 1}$) cross-peak at (11.0, 134) ppm for $\beta 37\text{Trp}$ disappears. The ($^1\text{H}_{\epsilon 1}$, $^{15}\text{N}_{\epsilon 1}$) cross-peak at (10.8, 129) ppm for $\beta 105\text{Trp}$ is much weaker in the presence of IHP at 29°C compared to that in the absence of IHP (Figures 17A and 17B). When the temperature is lowered in the
10 presence of IHP, the cross-peak at (11.0, 134) ppm reappears and two new cross-peaks appear at (11.0, 131) and (10.9, 130) ppm (Figures 17C and 17D). It appears that these two new cross peaks originate from $\beta 37\text{Trp}$ and $\beta 105\text{Trp}$.

Figures 18A and 18B show the ring-current-shifted proton resonances of Hb A, rHb (βL105w), rHb (αD94A , βL105W), and rHb (αD94A) in the CO form in the
15 absence and presence of IHP at 11, 20, and 29°C . The ring-current-shifted proton resonances of rHb (βL105W) in the CO form differ only slightly from those of Hb A in the absence of IHP, while they are very different from those of Hb A in the presence of IHP. The ring-current-shifted proton resonances of rHb (αD94A , βL105W) and rHb (αD94A) in the CO form are very different from those of Hb A in both the absence and
20 presence of IHP, but they are very similar to each other in the presence of IHP. In addition, the ring-current-shifted proton resonances of rHb (βL105W) in the CO form in the presence of IHP turn out to be very similar to those of rHb ($\alpha\text{D94A}/\beta\text{L105W}$) and

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rHb (α D94A) when the temperature is lowered (Figure 18B). It is believed that the spectra for the ring-current-shifted proton resonances of rHb (α D94A, β L105W) and rHb (α D94A) in the CO form in the presence IHP represent one type of spectrum for rHbs in the CO form with a stable T-structure. Therefore, the differences in heme
5 pocket conformations between mutant rHbs and Hb A also suggest that these mutant rHbs are much easier to switch from the R-structure to T-structure in light of the T-marker from the exchangeable proton resonances. The resonances at -1.8 and -1.9 ppm have been assigned to the heme pocket distal valine (E11) of α - and β -chains of Hb A, respectively (Dalvit, C., et al., Biochemistry 24: 3398 (1985) and Craescu, C.T., et al., Eur. J. Biochem 181: 87 (1989), the disclosures of which are incorporated herein by reference).
10 Compared to the spectra of Hb A, the resonance of distal valine (E11) of β -chain seems to be affected more in the spectra of mutant rHbs, especially in the presence of IHP, than that of α -chain (Figures 18A and 18B). These results imply that the structural switching from the R- to the T-structure induced by IHP, temperature, or the mutations
15 described herein might occur mainly in the β -chain.

Appropriately cross-linked rHb (β N108Q) and/or rHb (β L105W) can be incorporated into a hemoglobin-based blood substitute or therapeutic hemoglobin that is physiologically acceptable for use in clinical or veterinary medicine according to methods know in the art. See, for example, R.M. Winslow, et al. Eds. Blood Substitutes
20 Physiological Basis of Efficacy (Birkhauser, Boston, Mass.) (1995), the disclosure of which is incorporated herein by reference. The hemoglobin of the present invention

may also be advantageously used as a treatment for conditions such as septic shock, prevention of anaphylactic shock during dialysis.

Although the invention has been described in detail for the purposes of illustration, it is to be understood that such detail is solely for that purpose and that
5 variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention except as it may be limited by the claims.